



Dlx5 Is a Positive Regulator of Chondrocyte Differentiation during Endochondral Ossification

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The process of endochondral ossification in which the bones of the limb are formed after generation of cartilage models is dependent on a precisely regulated program of chondrocyte maturation. Here, we show that the homeobox-containing gene *Dlx5* is expressed at the onset of chondrocyte maturation during the conversion of immature proliferating chondrocytes into postmitotic hypertrophying chondrocytes, a critical step in the maturation process. Moreover, retroviral misexpression of *Dlx5* during differentiation of the skeletal elements of the chick limb *in vivo* results in the formation of severely shortened skeletal elements that contain excessive numbers of hypertrophying chondrocytes which extend into ectopic regions, including sites normally occupied by immature chondrocytes. The expansion in the extent of hypertrophic maturation detectable histologically is accompanied by expanded and upregulated domains of expression of molecular markers of chondrocyte maturation, particularly type X collagen and osteopontin, and by expansion of mineralized cartilage matrix, which is characteristic of terminal hypertrophic differentiation. Furthermore, *Dlx5* misexpression markedly reduces chondrocyte proliferation concomitant with promoting hypertrophic maturation. Taken together, these results indicate that *Dlx5* is a positive regulator of chondrocyte maturation and suggest that it regulates the process at least in part by promoting conversion of immature proliferating chondrocytes into hypertrophying chondrocytes. Retroviral misexpression of *Dlx5* also enhances formation of periosteal bone, which is derived from the *Dlx5*-expressing perichondrium that surrounds the diaphyses of the cartilage models. This suggests that *Dlx5* may be involved in regulating osteoblast differentiation, as well as chondrocyte maturation, during endochondral ossification. © 2002 Elsevier Science (USA)

Key Words: *Dlx5*; chondrocyte differentiation; chondrocyte maturation; chondrocyte hypertrophy; endochondral ossification; osteoblast differentiation; bone development; limb.

INTRODUCTION

The skeletal elements of the limb develop by a process called endochondral ossification in which bone is formed after the establishment of a cartilaginous template (Olsen *et al.*, 2000; de Crombrughe *et al.*, 2001; Wagner and Karsenty, 2001). At the onset of this process, mesenchymal cells in discrete regions of the developing limb bud aggregate into precartilage condensations which prefigure the future skeletal elements. The cartilage anlagen undergo rapid longitudinal and appositional growth as a result of proliferation and matrix deposition to form the cartilage models of the bones of the limb. The developing cartilage models become surrounded by a longitudinally oriented group of flattened cells called the perichondrium. Interac-

tions between the perichondrium and chondrocytes of the cartilage model play a major role in regulating various aspects of endochondral ossification (Long and Linsenmayer, 1998; Di Nino *et al.*, 2001).

Chondrocytes within the cartilage models progressively undergo a precisely regulated and coordinated program of maturation from proliferating to prehypertrophic to hypertrophic chondrocytes (Olsen *et al.*, 2000; de Crombrughe *et al.*, 2001; Wagner and Karsenty, 2001). Maturation commences in the central regions or diaphyses of the cartilage models where immature chondrocytes exit the cell cycle, enlarge, and are converted into prehypertrophic chondrocytes which are characterized by the expression of the secreted signaling molecule Indian hedgehog (Ihh) (Vortkamp *et al.*, 1996). The prehypertrophic chondrocytes undergo further differentiation to hypertrophic chondrocytes during which they continue to enlarge, initiate expression of type X collagen and other hypertrophic markers, includ-

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ing osteopontin and bone sialoprotein, and deposit a mineralized matrix. In parallel with the differentiation of hypertrophying chondrocytes, the perichondrium flanking the maturing chondrocytes becomes the periosteum, which is the source of osteogenic cells that deposit a bony collar around the diaphyses of the cartilage rudiments (Pechak *et al.*, 1986a). Blood vessels arising from the periosteum invade into the area of chondrocyte hypertrophy, a process dependent on vascular endothelial growth factor (VEGF) expressed by hypertrophic chondrocytes (Gerber *et al.*, 1999). Vascular invasion of the hypertrophic zone is accompanied by degradation and resorption of the mineralized cartilage matrix, its replacement by trabecular bone deposited by invading osteoblasts, and the concomitant excavation of the bone marrow cavity. The continued maturation of immature proliferating chondrocytes into hypertrophying chondrocytes and their replacement by trabecular bone and bone marrow results in the longitudinal growth of the long bones. Thus, a critical step in the longitudinal growth of long bones and endochondral ossification is the conversion of immature proliferating chondrocytes into hypertrophying chondrocytes. Indeed, hypertrophic maturation appears to be the main determinant of longitudinal growth (Hunziker, 1994).

The rate and progression of hypertrophic chondrocyte maturation during endochondral ossification is regulated by a coordinated balance between negative and positive signals. *Ihh* and parathyroid hormone-related peptide (PTHrP) function in a regulatory loop to negatively control chondrocyte maturation (Vortkamp *et al.*, 1996; Kronenberg *et al.*, 1997; St-Jacques *et al.*, 1999). In particular, *Ihh* expressed by newly postmitotic prehypertrophic chondrocytes induces *PTHrP* expression in the periarticular perichondrium adjacent to the immature proliferating chondrocytes in the epiphysis. In turn, PTHrP signals via its receptor PTH/PTHrP-R to suppress the maturation of proliferating chondrocytes in the subjacent epiphysis, thus maintaining a zone of immature chondrocytes competent to undergo proliferation (Vortkamp *et al.*, 1996; Kronenberg *et al.*, 1997; St-Jacques *et al.*, 1999). Members of the TGF- β family produced by the perichondrium have also been implicated as negative regulators of chondrocyte maturation (Serra *et al.*, 1997) and may act upstream of PTHrP to suppress the process (Serra *et al.*, 1999). In addition, the transition from prehypertrophic to hypertrophic chondrocytes is negatively controlled by Wnt-5a produced by the perichondrium (Hartmann and Tabin, 2000) and by the Notch receptor ligand Delta-1 produced by hypertrophic chondrocytes (Crowe *et al.*, 1999).

Recently, several positive regulators of chondrocyte maturation have been identified. These include one or more members of the BMP family (Enomoto-Iwamoto *et al.*, 1998; Volk *et al.*, 2000; Pathi *et al.*, 1999; Minina *et al.*, 2001), particularly BMP-6 (Grimsrud *et al.*, 1999; Ito *et al.*, 1999; Kameda *et al.*, 2000; Boskey *et al.*, 2002), which is specifically expressed by the prehypertrophic and hypertrophic chondrocytes (Kingsley, 1994; Vortkamp *et al.*, 1996;

Inada *et al.*, 1999). *Wnt-4* expressed by cells flanking the epiphysis of the cartilage models has also been implicated in the positive control of maturation (Hartmann and Tabin, 2000), and gain- and loss-of-function studies *in vitro* and *in vivo* have shown that the transcription factor *Cbfa1/Runx2* expressed by prehypertrophic and hypertrophic chondrocytes is an important positive regulator of the maturation process (Inada *et al.*, 1999; Kim *et al.*, 1999; Enomoto *et al.*, 2000; Takeda *et al.*, 2001; Ueta *et al.*, 2001; Leboy *et al.*, 2001).

Identification of the regulatory genes and signaling molecules that control the progression of chondrocyte maturation toward hypertrophy and the regulatory interactions among them is crucial to understanding normal and abnormal bone growth and development. In the present study, we provide evidence indicating that the homeodomain transcription factor *Dlx5* is an important positive regulator of chondrocyte maturation during endochondral ossification. *Dlx5* is one of six members of the *Dlx* family of homeobox-containing genes that are homologs of the *Drosophila Distal-less* gene, which is required for limb development in the fly embryo (Merlo *et al.*, 2000; Zerucha and Ekker, 2000). *Dlx5* has been implicated in several aspects of early vertebrate limb morphogenesis, including specification of the limb territories of the lateral plate, activity of the apical ectodermal ridge which directs limb outgrowth, and patterning along the anteroposterior axis (Ferrari *et al.*, 1995, 1999). Later in development, *Dlx5* is expressed by differentiating osteoblasts during intramembranous and endochondral ossification (Simeone *et al.*, 1994; Zhao *et al.*, 1994), and several studies suggest that it may play a role in osteoblast differentiation (Newberry *et al.*, 1998; Miyama *et al.*, 1999; Benson *et al.*, 2000; Tadic *et al.*, 2001, 2002).

Here, we report that *Dlx5* is expressed during the conversion of immature proliferating chondrocytes into postmitotic hypertrophying chondrocytes, which is a critical step in chondrocyte maturation during endochondral ossification. Moreover, retroviral misexpression of *Dlx5* during the differentiation of the skeletal elements of the chick limb *in vivo* promotes chondrocyte maturation, resulting in the formation of severely shortened skeletal elements that contain excessive numbers of hypertrophying chondrocytes. Our results indicate that *Dlx5* positively regulates chondrocyte maturation, and may do so at least in part by promoting conversion of immature proliferating chondrocytes to hypertrophying chondrocytes.

MATERIALS AND METHODS

Preparation of *Dlx5* Retroviral Expression Vector and Infection Protocol

The methods used for the preparation and propagation of the avian replication competent *Dlx5* expression vector were essentially the same as those previously described (Ferrari *et al.*, 1998) and follow standard established protocols (Morgan and Fekete, 1996). Briefly, our cDNA containing the full coding sequence of

chicken *Dlx5* (Ferrari *et al.*, 1995) was cloned into the *Cla*I site of RCASBP(A), a derivative of Rous Sarcoma Virus (RSV) containing a portion of the *pol* gene from the Bryan high titer strain of RSV (obtained from Steve Hughes). Chick embryo fibroblasts (CEF) were transfected with the retroviral plasmid DNA, and the cells were cultured until virus production was high as assayed by media reverse transcriptase activity (Ferrari *et al.*, 1998). Concentrated retroviral stocks were prepared by ultracentrifugation of the virus-containing media (Morgan and Fekete, 1996; Ferrari *et al.*, 1998), and aliquots were rapidly frozen. Titters were determined by adding limiting dilutions of the concentrated retroviral stock to subconfluent CEF cultures, immunostaining with a monoclonal antibody against the RSV viral *gag* antigen p19, and counting the number of positive colonies (Morgan and Fekete, 1996; Ferrari *et al.*, 1998). Titters ranged from 0.5 to 1×10^9 cfu/ml.

Concentrated *Dlx5* retrovirus or control RCASBP(A) retrovirus lacking the *Dlx5* cDNA insert was microinjected as previously described (Ferrari *et al.*, 1998) into the proximal posterior portion of stage 21/22 right chick wing buds, which will give rise primarily to the humerus, ulna, and radius. This resulted in widespread *Dlx5* expression detectable by *in situ* hybridization throughout the proximal skeletal elements and soft tissue of the infected limbs (data not shown). Limbs infected with control retrovirus exhibited no detectable abnormalities.

Skeletal Staining and Histology

The skeletons of day 13–18 embryos were whole-mount stained with Alcian blue and Alizarin Red to visualize cartilage and mineralized bone, respectively. Older embryos were usually eviscerated before staining. Briefly, embryos were incubated in 0.015% Alcian blue in 95% ethanol/20% glacial acetic acid for 2–4 days, after which they were cleared for 2–4 days in 2% KOH. They were then stained overnight in 0.1% Alizarin Red S in 1% KOH followed by clearing in 0.5% KOH and a graded series of KOH/glycerine solutions to 100% glycerine. Infected and contralateral control limbs were removed in 100% glycerine and photographed. After photography, some of the limbs were washed in PBS, dehydrated in 100% ethanol, cleared in xylene, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

In Situ Hybridization

In situ hybridization on serially sectioned limbs that had been fixed in Bouin's solution was performed by using 33 P-labeled cDNA probes (see below) and high-stringency hybridization conditions as previously described (Coelho *et al.*, 1991). In order to carefully correlate expression patterns of different genes or to compare different parameters, in many cases, adjacent sections (within 20 μ m of one another) of the same specimens were mounted on separate slides and hybridized with different probes and/or subjected to different analyses (see below).

The cDNA probes used for *in situ* hybridization were as follows: a 323-bp probe from the 3' untranslated region of chicken *Dlx5* cDNA prepared as previously described (Ferrari *et al.*, 1999); a 560-bp probe from the 5' end of chicken *Ihh* cDNA prepared by digesting clone pciHHR4–1 Sac (provided by Cliff Tabin) (Vortkamp *et al.*, 1996) in Bluescript SK+ with *Eco*RI and *Sac*I; a 234-bp probe from the 5' end of chicken *PTHrP* cDNA prepared by digesting clone PTHrP2 (provided by Cliff Tabin) (Vortkamp *et al.*, 1996) in pGEMT with *Nco*I and *Spe*I; a 1.1-kb probe corresponding to the full coding sequence of chicken *osteopontin* cDNA prepared

by digesting clone pMMPP2 (provided by Louis Gerstenfeld) (Moore *et al.*, 1991) in Bluescript SK with *Eco*RI.

The cDNA probes were labeled with [33 P]dCTP by the random oligonucleotide primer procedure.

Immunohistochemistry

Immunostaining for type X collagen with monoclonal antibody X-AC9 (Schmid and Linsenmayer, 1985) (Developmental Studies Hybridoma Bank) on serially sectioned limbs that had been fixed in Bouin's solution was carried out as previously described (Kosher and Solursh, 1989) by using a Vectastain Elite ABC Kit. Sections were treated with 5 mg/ml bovine testicular hyaluronidase (Type I-S; Sigma) prior to immunostaining, and staining was done with a 1:80 dilution of X-AC9 in PBS containing 5% normal horse serum.

Histochemical Analysis of Mineralization

For histochemical detection of mineralized matrix, serially sectioned limbs that had been fixed in 95% ethanol were stained with 0.5% Alizarin Red S, pH 4.0, for 5 min at room temperature as described by Yagami *et al.* (1999).

Analysis of Cell Proliferation

Cell proliferation was assayed immunohistochemically by staining serially sectioned limbs that had been fixed in Bouin's solution for PCNA (proliferating cell nuclear antigen), an accessory protein for DNA polymerase that is present in the nuclei of dividing cells (Hall *et al.*, 1990; Waseem and Lane, 1990), using a monoclonal antibody against PCNA (clone PC10) and a kit obtained from Novacastra. Before immunostaining, sections were treated with Vector antigen unmasking solution at 95° for 5 min, and after immunostaining, the sections were lightly counterstained with hematoxylin to detect unlabeled nuclei. The number of PCNA labeled and unlabeled nuclei in four adjacent sections through the middle of the metaphyseal region of *Dlx5*-infected and contralateral control limbs were counted in photomicrographs.

RESULTS

Dlx5 Is Expressed at the Onset of Chondrocyte Maturation Concomitant with the Conversion of Immature Proliferating Chondrocytes into Hypertrophying Chondrocytes

After the formation of the cartilage models of the long bones of the limb, *Dlx5* is highly expressed by the perichondrium adjacent to the diaphysis of the models that will give rise to the osteoblasts that deposit the periosteal bony collar (Fig. 1). As endochondral ossification proceeds, *Dlx5* is indeed very highly expressed in the inner layers of the periosteum where osteoblasts are differentiating and depositing periosteal bone (data not shown). These observations suggest the possibility that *Dlx5* may be involved in regulating the onset of periosteal bone differentiation during endochondral ossification.

In addition to being expressed by the perichondrium/periosteum, *Dlx5* is expressed by the maturing prehypertro-

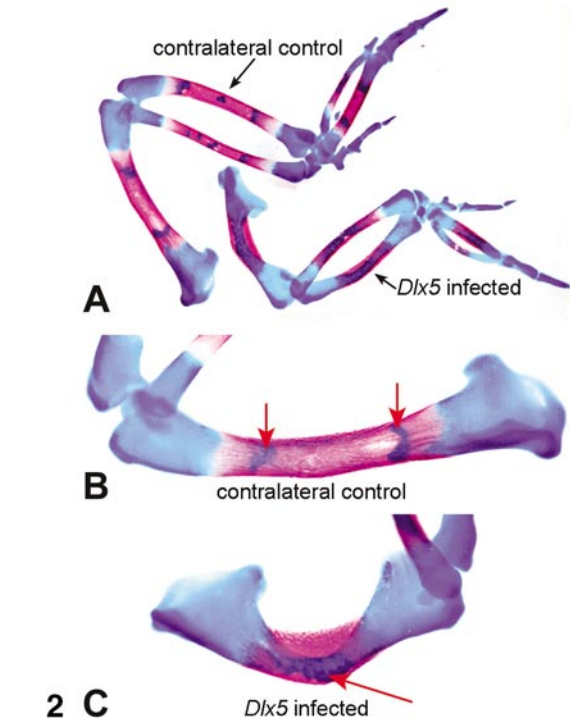
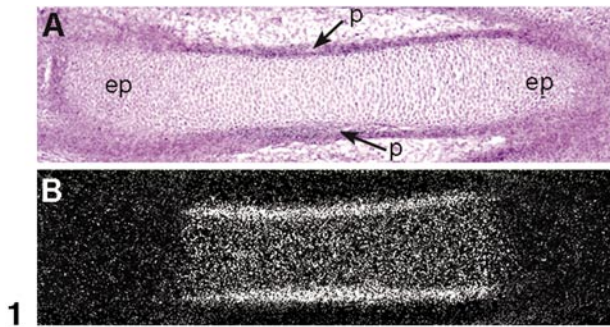


FIG. 1. Expression of *Dlx5* in a metacarpal rudiment at day 6. (A) Bright field. (B) Dark field. *Dlx5* is expressed in the perichondrium (p, arrows) adjacent to the diaphysis of the cartilage models that will give rise to the periosteal bony collar. *Dlx5* is also expressed by the maturing prehypertrophic and hypertrophic chondrocytes in the diaphysis of the cartilage models, but not by the immature chondrocytes in the epiphyses (ep).

FIG. 2. (A–C) Alcian blue/Alizarin Red-stained skeletal elements of day 13 contralateral control and *Dlx5* infected limbs. The control and *Dlx5* infected humerus rudiments are shown at higher magnification in (B, C). The humerus rudiments of the *Dlx5*-infected limbs are severely shortened and characteristically misshapen and bent (C). The ulna and the radius of the infected limbs are also shorter, but the distal skeletal elements are little affected. Also note that narrow zones of chondrocytes that stain very darkly with Alcian blue are present adjacent to the marrow cavity in control skeletal elements (red arrows in B). In the *Dlx5*-infected rudiments, the zone of darkly staining chondrocytes is expanded and extends deeply into the marrow cavity (red arrow in C).

phic and hypertrophic chondrocytes in the central regions or diaphyses of the cartilage models (Fig. 1). In contrast, *Dlx5* is not expressed by the immature proliferating chondrocytes in the epiphyses (Fig. 1). Thus, *Dlx5* expression in the cartilage models is initiated concomitant with the conversion of immature proliferating chondrocytes into postmitotic prehypertrophic chondrocytes, which is a critical step in chondrocyte maturation. This pattern of expression is suggestive of a possible role for *Dlx5* in the regulation of chondrocyte maturation during endochondral ossification.

***Dlx5* Misexpression during Skeletal Differentiation *In Vivo* Results in the Formation of Severely Shortened Skeletal Elements**

To explore the role of *Dlx5* in endochondral ossification, we misexpressed *Dlx5* during the differentiation of the skeletal elements of the developing chick limb *in ovo* with the avian replication competent retroviral vector RCASBP to determine whether misexpression of *Dlx5* modified the process of chondrocyte maturation or other aspects of endochondral ossification. In these studies, we microinjected concentrated *Dlx5* retrovirus into the proximal posterior portions of stage 21/22 (day 4) right chick wing buds, which will give rise primarily to the humerus, ulna, and radius. At various times after microinjection, the skeletons of the embryos were stained with Alcian blue and Alizarin Red to visualize cartilage and mineralized bone, respectively.

At day 13 (Fig. 2) and day 18 (Fig. 3), the proximal skeletal elements, particularly the humerus rudiments of the *Dlx5* infected limbs are severely shortened. The humerus rudiments of the *Dlx5* infected limbs are on average $30.3 \pm 3.2\%$ shorter than their corresponding uninjected contralateral control limbs at day 13 and $45.6 \pm 2.8\%$ shorter at day 18. Furthermore, the length of the *Dlx5*-infected humerus rudiments increases by only 13% between days 13 and 18, whereas the length of the control rudiments increases by 50% during the same period. The radius and particularly the ulna rudiments of the *Dlx5*-infected limbs are also shorter than controls (ulna and radius $30.6 \pm 2.1\%$ and $18.2 \pm 2.8\%$ shorter, respectively on E18), although not to quite the same extent as the humerus, whereas the distal skeletal elements are little affected ($91.2 \pm 2.1\%$ the length of controls on E18). This proximodistal difference in severity of effect is not unexpected, and reflects the fact that the *Dlx5* retrovirus was initially microinjected into the prospective proximal portions of the limb bud at stage 21/22. Consistent with this, when *Dlx5* retrovirus is injected throughout the distal posterior portion of the limb bud at an earlier developmental stage (stage 17), shortening of the digits ($29.7 \pm 3.4\%$ shorter than controls at E13) as well as more proximal skeletal elements is detected (data not shown). These results clearly indicate that misexpression of *Dlx5* inhibits the longitudinal growth of developing long bones.

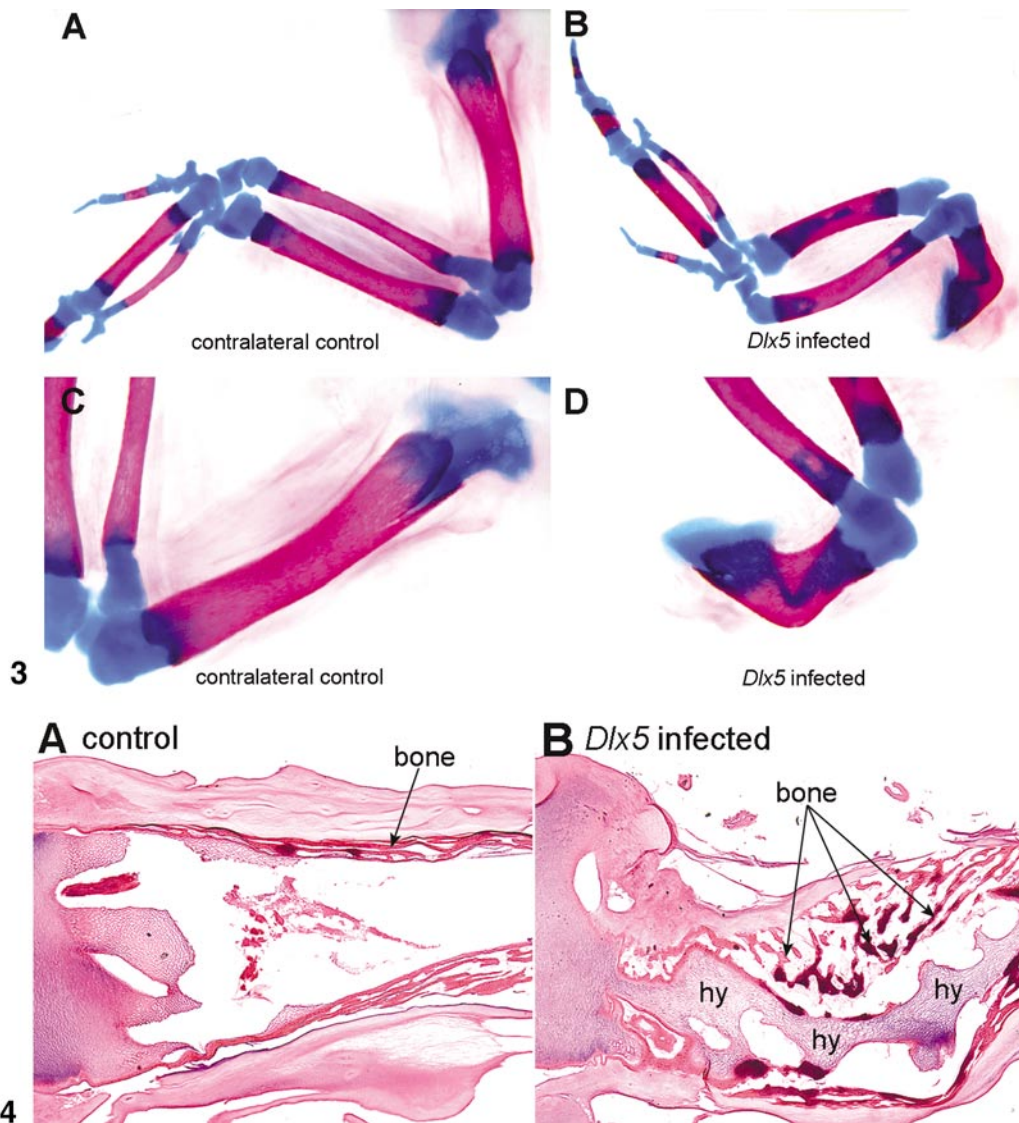


FIG. 3. (A, B) Alcian blue/Alizarin Red-stained skeletal elements of day 18 contralateral control (A) and *Dlx5*-infected (B) limbs. (C, D) Higher magnification images of the control (C) and *Dlx5*-infected (D) humerus rudiments. The humerus rudiments of the *Dlx5*-infected limbs are severely shortened and characteristically misshapen and bent (D). The ulna and the radius of the infected limbs are also shorter, but the distal skeletal elements are little affected.

FIG. 4. Hematoxylin and eosin-stained sections through a contralateral control (A) and *Dlx5*-infected (B) humerus at day 18. The *Dlx5*-infected humerus (B) contains an excessive number of enlarged rounded apparently hypertrophic chondrocytes (hy) that extend deeply into the marrow cavity. There is also an increase in the amount of periosteal bone (bone) along the diaphysis of the *Dlx5*-infected humerus.

Interestingly, the humerus rudiments of the *Dlx5*-infected limbs are not only considerably shorter, but are also characteristically misshapen and bent (Figs. 2 and 3). Although the reason for this characteristic bending is not clear, it is possible that the impaired elongation of the *Dlx5* infected humerus rudiments coupled with normal growth of the surrounding nonskeletal tissues such as tendons might result in such misshaping.

It is noteworthy that a narrow zone of chondrocytes that

stain very darkly with Alcian blue is present adjacent to the marrow cavity in control skeletal elements (arrows in Fig. 2B). Significantly, in the *Dlx5*-infected skeletal elements, the zone of darkly staining chondrocytes is greatly expanded and extends deeply into the marrow cavity (arrow in Figs. 2C). As described below, these darkly staining zones consist of terminally differentiated hypertrophic chondrocytes detectable histologically and histochemically.

It is also noteworthy that the Alizarin Red-stained peri-

osteal bony collars that form around the diaphyses of the *Dlx5*-infected skeletal elements are broader than controls, particularly at day 18 (Fig. 3). The Alizarin Red-stained periosteal bony collars are $53 \pm 11\%$ wider in the *Dlx5*-infected humerus rudiments than in contralateral controls, and the bony collars of the *Dlx5*-infected ulna and radius rudiments are $32 \pm 1.6\%$ and $22 \pm 2\%$ wider than controls, respectively. So, although *Dlx5* misexpression inhibits longitudinal growth, it does not inhibit periosteal bone formation, and in fact seems to stimulate it (see below).

The Shortened Skeletal Elements Formed in Response to Dlx5 Misexpression Contain Excessive Numbers of Hypertrophying Chondrocytes

To gain further insight into the mechanism by which *Dlx5* misexpression inhibits the longitudinal growth of developing long bones, control and *Dlx5*-infected skeletal elements were sectioned and the histology of the skeletal rudiments was examined after staining with hematoxylin and eosin. As shown in Fig. 4, *Dlx5*-infected skeletal elements contain an excessive number of enlarged rounded cells morphologically resembling hypertrophying chondrocytes. Whereas control humerus rudiments have a relatively small zone of large rounded hypertrophic chondrocytes adjacent to the marrow cavity, in the *Dlx5*-infected rudiments, the zone of apparently hypertrophic chondrocytes is greatly expanded and extends deeply into the marrow cavity of the diaphysis (Fig. 4). Furthermore, as shown at high magnification in Fig. 5, in severely affected *Dlx5*-infected skeletal elements, enlarged rounded cells clearly resembling hypertrophying chondrocytes extend into the metaphyseal and proximal epiphyseal regions that are normally occupied by immature proliferating chondrocytes. These regions of contralateral control skeletal elements consist of adjacent organized groups of small rounded and radially flattened chondrocytes which represent immature proliferating chondrocytes (Fig. 5) (Hartmann and Tabin, 2000). In contrast, the metaphyseal and proximal epiphyseal regions of severely affected *Dlx5* rudiments contain relatively large numbers of enlarged rounded cells morphologically resembling hypertrophying chondrocytes and lack the organized groups of small rounded and radially flattened putative immature proliferating chondrocytes that are present throughout the corresponding regions of controls (Fig. 5). These histological analyses suggest that chondrocyte maturation is promoted as a consequence of *Dlx5* misexpression.

Although ectopic hypertrophic chondrocytes are present in the proximal epiphyseal and metaphyseal region of *Dlx5*-infected skeletal elements, they are rarely observed in the epiphyseal region at the articular ends of the rudiments. This suggests the interesting possibility that the chondrocytes directly subjacent to the periarticular perichondrium are refractory or less sensitive to *Dlx5* misexpression than the immature proliferating chondrocytes in more proximal regions of the epiphysis and in the metaphysis.

It is also noteworthy that there is an obvious increase detectable histologically in the amount of periosteal bone that is present along the diaphysis of the *Dlx5*-infected humerus rudiments compared with contralateral controls (Fig. 4), and this apparently accounts for the increased thickness of the Alizarin Red-stained periosteal bony collar detectable by whole-mount staining in the *Dlx5* limbs.

Dlx5 Misexpression during Skeletal Differentiation in Vivo Results in Expanded Domains of Expression of Markers of Chondrocyte Maturation

To confirm the apparent striking expansion in the extent of hypertrophic maturation detectable histologically in response to *Dlx5* misexpression and to gain further insight into the mechanism of *Dlx5* action during endochondral ossification, we examined the effect of *Dlx5* misexpression on the expression of molecular markers of hypertrophic chondrocyte maturation particularly osteopontin and type X collagen. Osteopontin is a late marker of chondrocyte maturation which is characteristically expressed by terminally differentiated hypertrophic chondrocytes that are undergoing mineralization (Gerstenfeld and Shapiro, 1996; Iwamoto *et al.*, 1993; Koyama *et al.*, 1999). At day 13 (Fig. 6) and day 18 (Fig. 7), the expression domain of *osteopontin* detectable by *in situ* hybridization is greatly expanded in the *Dlx5*-infected skeletal elements compared with uninjected contralateral controls. The length of the *osteopontin* expression domain in the *Dlx5*-infected humerus rudiment is expanded by about twofold compared with contralateral controls. Not only is the expression domain of *osteopontin* expanded, but there is also a striking increase in the intensity of the *osteopontin* hybridization signal in the *Dlx5*-infected elements compared with controls (Fig. 6). A similar expansion in the expression domain of type X collagen, a definitive molecular marker of hypertrophic chondrocytes, is present in the *Dlx5*-infected skeletal elements (Fig. 7). Thus, the apparent expansion in the extent of hypertrophic maturation detectable histologically in response to *Dlx5* misexpression is accompanied by expanded and apparently upregulated domains of expression of molecular markers of hypertrophic chondrocyte maturation.

During terminal phases of maturation, the matrix surrounding hypertrophic chondrocytes becomes progressively mineralized. To further examine the effect of *Dlx5* misexpression on maturation, sections through *Dlx5*-infected and control skeletal elements were stained with Alizarin Red which specifically binds to mineralized matrix. In day 13 control skeletal elements, Alizarin Red-stainable mineralized cartilage matrix is limited to small regions of terminally differentiated hypertrophic chondrocytes near the bone marrow cavity (Fig. 8). As shown in Fig. 8, the region of Alizarin Red-stained mineralized cartilage matrix is greatly expanded in *Dlx5*-infected skeletal elements.

To study further the effects of *Dlx5* on the regulation of chondrocyte maturation, the expression domains of *Ihh* and *PTHrP* were examined in control and *Dlx5*-infected skeletal

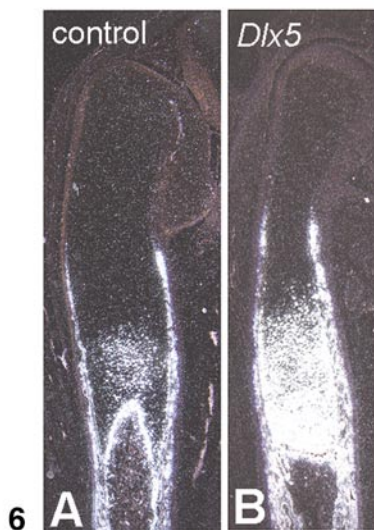
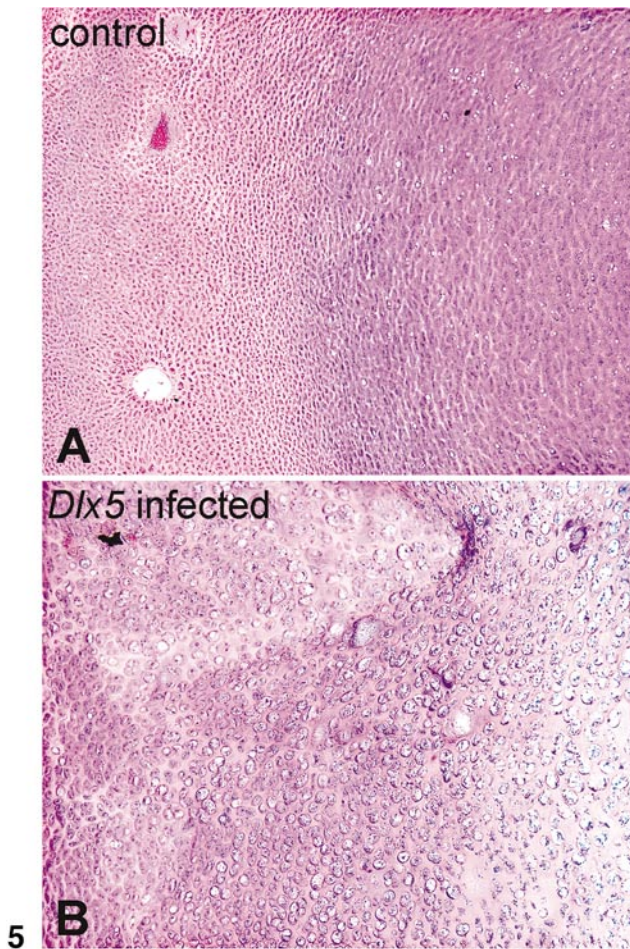


FIG. 5. High-magnification images of hematoxylin and eosin-stained sections through the proximal epiphyseal and metaphyseal regions of day 18 control (A) and *Dlx5*-infected (B) humerus rudiments. This region of the control rudiment (A) consists of adjacent organized zones of small rounded (toward the left) and

elements. Although at day 13 there is an expansion in the *Ihh* expression domain in *Dlx5*-infected skeletal elements, the expression domain of *PTHrP* in the periarticular perichondrium appears normal, and no ectopic expression of *PTHrP* is detectable in the *Dlx5*-infected elements (Fig. 9). Thus, *Dlx5* does not appear to interfere with the *Ihh*/*PTHrP* regulatory loop that negatively controls the progression of proliferating chondrocytes to prehypertrophic chondrocytes.

As described above, in addition to enhanced chondrocyte maturation, there is an increase in thickness of the periosteal bony collar along the diaphyses of the *Dlx5* infected skeletal elements compared with contralateral controls detectable by whole-mount staining (Fig. 3) and histologically (Fig. 4). As shown in Fig. 10, the increase in the amount of mineralized periosteal bone in response to *Dlx5* misexpression is also clearly evident in Alizarin Red-stained tissue sections, and is also reflected in an expanded periosteal domain of expression of *osteopontin*, which is a marker of differentiated osteoblasts as well as a late marker of chondrocyte maturation. These results further suggest that *Dlx5* may be involved directly or indirectly in regulating osteoblast differentiation during the formation of the periosteal bony collar, as well as in regulating chondrocyte maturation.

***Dlx5* Misexpression Markedly Reduces Chondrocyte Proliferation as It Promotes Maturation**

At the onset of chondrocyte maturation, immature proliferating chondrocytes exit the cell cycle concomitant with initiating hypertrophic differentiation. As described above, our histological analyses indicated that, in addition to an enhancement of hypertrophic maturation, there was an apparent decrease in the size of the zone of proliferation in response to *Dlx5* misexpression. In particular, *Dlx5*-infected skeletal elements lack the organized groups of rounded and radially flattened chondrocytes that constitute the zones of proliferation in the metaphyseal regions of control skeletal elements. To confirm and quantify the apparent reduction in proliferation detectable histologically in response to *Dlx5* misexpression, we compared the expression of PCNA (proliferating cell nuclear antigen) by

radially flattened (toward the right) immature proliferating chondrocytes. The corresponding region of the *Dlx5*-infected rudiment (B) contains large numbers of enlarged rounded cells resembling hypertrophying chondrocytes. The images of the control and *Dlx5*-infected rudiments are at equivalent distances (about 0.5 mm) from the articular surfaces.

FIG. 6. Osteopontin expression in a contralateral control (A) and *Dlx5*-infected (B) humerus rudiment at day 13. The *osteopontin* expression domain is expanded in the *Dlx5*-infected humerus and the hybridization signal is considerably more intense.

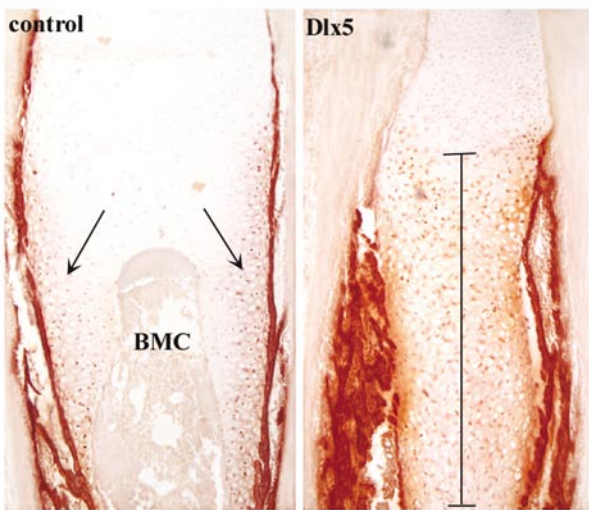
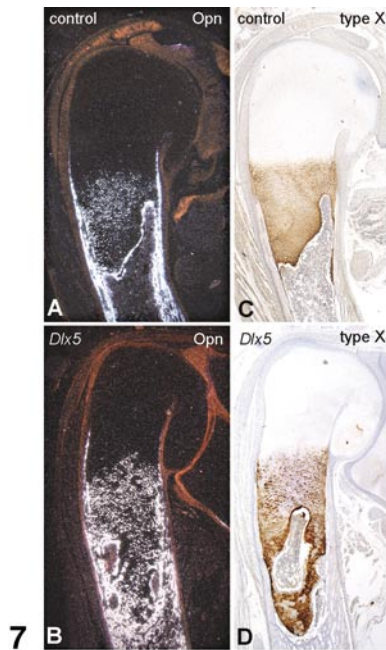


FIG. 7. (A, B) *Osteopontin* (Opn) expression detectable by *in situ* hybridization, and (C, D) type X collagen expression detectable by immunostaining in adjacent sections of a contralateral control (A, C) and *Dlx5*-infected (B, D) humerus rudiment at day 18. The *osteopontin* and type X collagen expression domains are expanded in the *Dlx5*-infected rudiment.

FIG. 8. Mineralized cartilage matrix detectable by Alizarin Red staining in a contralateral control and *Dlx5*-infected humerus rudiment at day 13. In the control, mineralized cartilage matrix (arrows) is limited to small regions near the bone marrow cavity (BMC). In the *Dlx5*-infected rudiment, the region of mineralized cartilage matrix (bar) is greatly expanded.

immunohistochemistry in *Dlx5*-infected and control humerus elements. PCNA is an accessory protein for DNA polymerase that is synthesized in the late G₁ and S phases

of the cell cycle and thus is specifically present in the nuclei of dividing cells (Hall *et al.*, 1990; Waseem and Lane, 1990).

As shown in Fig. 11, there is a striking reduction in the number of PCNA-positive nuclei in the metaphyseal region of *Dlx5*-infected skeletal elements compared with uninfected contralateral controls. Most of the nuclei of the rounded and radially flattened immature chondrocytes in the zone of proliferation in the metaphyseal region of control skeletal elements are PCNA-positive. In contrast, the metaphyseal region of the *Dlx5*-infected elements contains far fewer PCNA-positive nuclei, and the majority of the nuclei are unlabeled (Fig. 11). There is on average a 44.3% reduction in the proportion of dividing (PCNA-positive) cells in the metaphyseal region of the *Dlx5* infected elements compared with contralateral controls. Whereas $69.3 \pm 1.9\%$ of the nuclei in the proliferation zone of control humerus elements are PCNA-positive, only

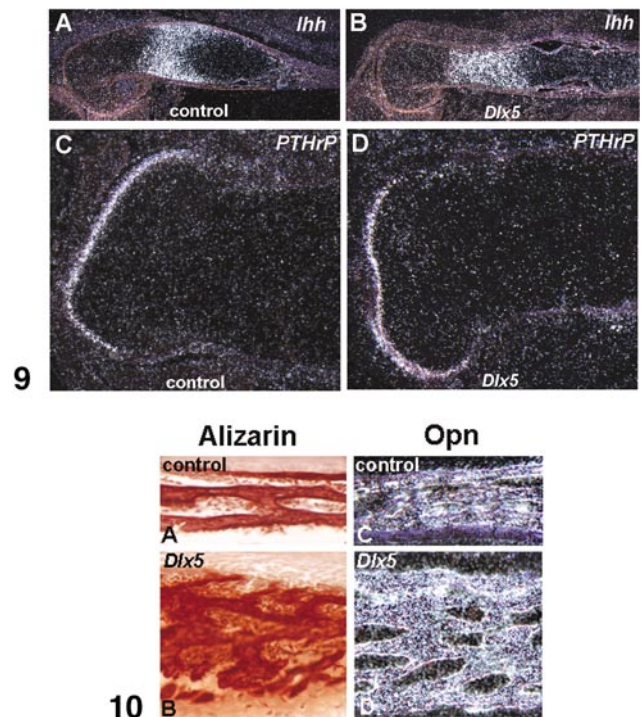


FIG. 9. (A, B) *Ihh* and (C, D) *PTHrP* expression in contralateral control (A, C) and *Dlx5*-infected (B, D) humerus rudiments at E13. Although there is an expansion in the *Ihh* expression domain in the *Dlx5*-infected rudiment, the expression of *PTHrP* in the periarticular perichondrium is unaffected.

FIG. 10. (A, B) Alizarin Red-stained periosteal bony collars in control (A) and *Dlx5*-infected (B) humerus rudiments at day 13. There is a striking increase amount of mineralized periosteal bone in the *Dlx5*-infected rudiment. (C, D) *Osteopontin* (Opn) expression in the periosteal bony collars of control (C) and *Dlx5*-infected (D) humerus rudiments. The increase in the amount of periosteal bone in the infected rudiment is reflected in expanded *osteopontin* expression.

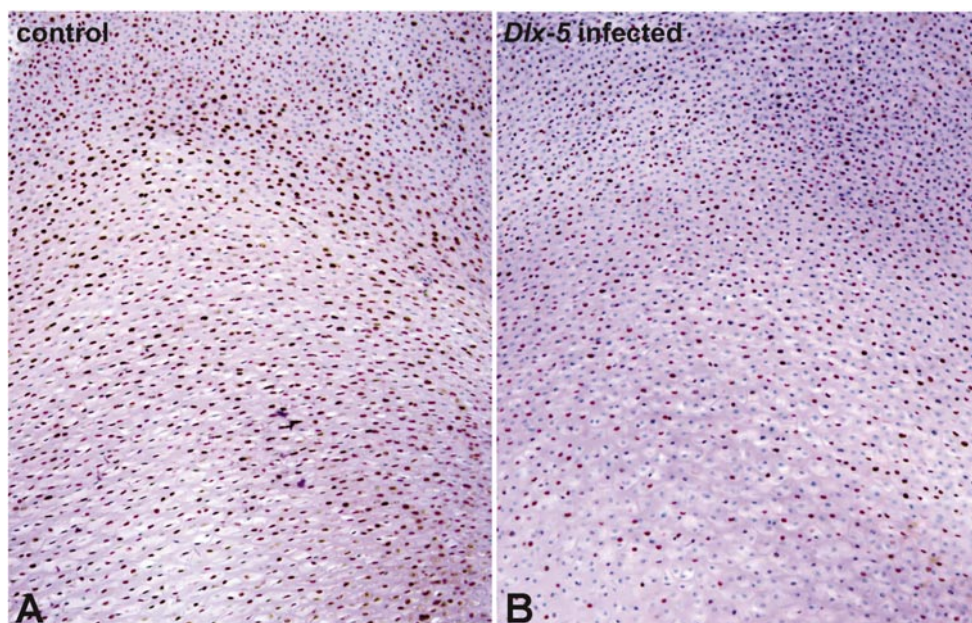


FIG. 11. Cell proliferation detectable by PCNA immunohistochemistry in the metaphyseal region of a control (A) and *Dlx5*-infected (B) humerus rudiment at day 18. There is a striking reduction in the number of PCNA-positive nuclei in the *Dlx5*-infected rudiment. On average, there is about a 44% reduction in the proportion of dividing (PCNA positive) cells in the *Dlx5*-infected elements compared with contralateral controls (see text for details).

$38.6 \pm 2.6\%$ of the nuclei in the metaphyseal region of the *Dlx5*-infected elements are labeled. The difference in proliferation in *Dlx5*-infected and control elements is most pronounced in the lower portion of the metaphysis, where $95.9 \pm 0.9\%$ of the radially arranged chondrocytes in control elements are proliferating, whereas only $30.5 \pm 2.7\%$ of the enlarged chondrocytes in the corresponding region of *Dlx5* infected rudiments are dividing. Thus, *Dlx5* misexpression markedly reduces chondrocyte proliferation as it promotes chondrocyte maturation.

DISCUSSION

Dlx5 Is a Positive Regulator of Chondrocyte Maturation during Endochondral Ossification

The process of endochondral ossification in which the bones of the limb are formed after the generation of cartilage models is dependent on a precisely regulated and coordinated program of chondrocyte maturation. A crucial event in chondrocyte maturation is differentiation of immature proliferating chondrocytes into postmitotic prehypertrophic chondrocytes which then undergo hypertrophy. Hypertrophic maturation appears to be the main determinant of longitudinal growth (Hunziker, 1994). Identification of regulatory genes that control the progression of chondrocyte maturation toward hypertrophy is crucial to understanding normal and abnormal bone growth and development.

The results of the present study indicate that the homeodomain transcription factor *Dlx5* is an important positive regulator of chondrocyte maturation in the developing skeletal elements of the limb. First, we demonstrate that *Dlx5* is expressed at the onset of chondrocyte maturation during the conversion of immature proliferating chondrocytes into hypertrophying chondrocytes, which is a critical step in the maturation process. Moreover, misexpression of *Dlx5* during the differentiation of the skeletal elements of the chick limb *in vivo* promotes chondrocyte maturation resulting in the formation of severely shortened skeletal elements that contain excessive numbers of hypertrophying chondrocytes extending into ectopic regions. In response to *Dlx5* misexpression, ectopic hypertrophying chondrocytes not only extend deeply into the marrow cavity, but also are present in the metaphyseal and proximal epiphyseal regions that are normally occupied by immature proliferating chondrocytes. The expansion in the extent of hypertrophic maturation in response to *Dlx5* misexpression is accompanied by expanded domains of expression of molecular markers of hypertrophic chondrocyte maturation, particularly osteopontin and type X collagen, and also by an expansion of mineralized cartilage matrix, which is characteristic of terminal hypertrophic differentiation. Finally, *Dlx5* misexpression markedly reduces chondrocyte proliferation concomitant with promoting hypertrophic maturation.

Taken together, these results indicate that *Dlx5* is an important positive regulator of chondrocyte maturation during endochondral ossification and suggest that it may

regulate maturation at least in part by promoting the conversion of immature proliferating chondrocytes into hypertrophying chondrocytes. This possibility is consistent with our finding that during normal development *Dlx5* expression is initiated concomitant with the conversion of immature proliferating chondrocytes into postmitotic prehypertrophic chondrocytes.

Since our gain-of-function study clearly implicates *Dlx5* in the regulation of chondrocyte maturation, it is of considerable interest that *Dlx5*-null mice exhibit no obvious defects in the longitudinal growth of long bones or malformations of the limbs (Acampora *et al.*, 1999; Depew *et al.*, 1999), although there is a subtle reduction of the periosteal bone lamina and defects in craniofacial skeletogenesis (Acampora *et al.*, 1999). In this regard, it is noteworthy that *Dlx6*, a closely related and potentially functionally redundant member of the *Dlx* family, has been reported to be coexpressed with *Dlx5* during skeletogenesis (Simeone *et al.*, 1994; Acampora *et al.*, 1999; Chen *et al.*, 1996). Thus, in the absence of *Dlx5*, *Dlx6* or some other member of the *Dlx* family may compensate for *Dlx5* in regulating chondrocyte maturation. It is therefore of considerable interest that it has recently been demonstrated that chondrocyte maturation is severely inhibited and retarded in mouse embryos in which the *Dlx5* and *Dlx6* genes have been simultaneously inactivated (Robledo *et al.*, 2002). This loss-of-function study complements and confirms our gain-of-function study, indicating that *Dlx5* and other *Dlx* genes are positive regulators of chondrocyte maturation during endochondral ossification. The *Dlx5/Dlx6* double inactivation study also indicates that *Dlx5*, *Dlx6*, and perhaps other members of the *Dlx* family function in a redundant or concerted fashion to regulate chondrocyte maturation.

Although our results clearly indicate *Dlx5* is a positive regulator of chondrocyte maturation, the extension of hypertrophic chondrocytes into the marrow cavity in response to *Dlx5* misexpression might also reflect, at least in part, alterations in mechanisms of remodeling or removal of the hypertrophic zone. Alternatively, the rate of removal of the hypertrophic chondrocytes in the marrow cavity might not be able to keep pace with the enhanced rate of hypertrophic maturation that occurs in response to *Dlx5* misexpression. In any case, the possibility that *Dlx5* may be involved in remodeling as well as in chondrocyte maturation warrants consideration.

Possible Relationships between *Dlx5* and Other Regulators of Chondrocyte Maturation during Endochondral Ossification

In addition to *Dlx5*, several other regulatory molecules, including BMPs, particularly BMP-6, the transcription factor *Cbfa1/Runx2*, and β -catenin-mediated Wnt signaling, have been implicated in the positive regulation of chondrocyte maturation during endochondral ossification (see Introduction). Unraveling the ways in which *Dlx5* and other

transcription factors and signaling pathways interact and cooperate with one another to regulate the progression of hypertrophic chondrocyte maturation is critical to understanding the differentiation and growth of the skeletal elements of the limbs. Although the relationships between *Dlx5* and the other positive regulators of maturation remain to be determined, previous studies are suggestive of several possible regulatory interactions.

Like *Dlx5*, BMP-6, which can stimulate chondrocyte maturation *in vitro* (Grimsrud *et al.*, 1999; Ito *et al.*, 1999; Kameda *et al.*, 2000; Boskey *et al.*, 2002), is expressed in prehypertrophic and hypertrophic chondrocytes *in vivo* (Kingsley, 1994; Vortkamp *et al.*, 1996; Inada *et al.*, 1999). It is therefore noteworthy that *Dlx5* is a direct downstream target gene of BMP signaling in osteoblasts (Miyama *et al.*, 1999) and other cell types (Luo *et al.*, 2001; Park and Morasso, 2002). Thus, it is possible that *Dlx5* expression may be regulated by BMP-6 or another member of the BMP family during chondrocyte maturation. If so, *Dlx5* may participate in mediating at least some of the positive effects of BMP signaling on hypertrophic differentiation. It is also possible that *Dlx5* and BMP-activated Smad transcription factors, such as Smads 1, 5, or 8, might function as coactivators of the transcription of some target genes during chondrocyte maturation. Both homeodomain binding elements and BMP responsive Smad binding elements are involved in regulation of the *osteopontin* gene promoter in osteoblasts (Hullinger *et al.*, 2001), and the *osteopontin* gene is a candidate direct target of *Dlx5* during chondrocyte maturation, since its expression domain is expanded and upregulated in response to *Dlx5* misexpression.

Cbfa1/Runx2, another positive regulator of chondrocyte maturation, is coexpressed with *Dlx5* and BMP-6 by prehypertrophic and hypertrophic chondrocytes of the cartilage models (Inada *et al.*, 1999; Kim *et al.*, 1999), and the skeletal elements of *Cbfa1/Runx2* knockout mice in which chondrocyte maturation is suppressed do not express BMP-6 (Inada *et al.*, 1999). Thus, it is possible that *Cbfa1/Runx2* might be an upstream regulator of BMP-6 expression at the onset of hypertrophic differentiation, and, in turn, as described above, BMP signaling may regulate the expression of *Dlx5*. Conversely, several studies have shown that BMP signaling can upregulate the expression of *Cbfa1/Runx2* in osteoblasts (Chen *et al.*, 1998; Lee *et al.*, 1999, 2000; Gori *et al.*, 1999; Bannerjee *et al.*, 2001) and in a chondrocyte-like cell line, TC6 (Takazawa *et al.*, 2000). Thus, BMP signaling has the potential to regulate expression of both *Dlx5* and *Cbfa1/Runx2* during chondrocyte maturation, and these transcription factors may then function separately or work together as comodulators of some target genes in chondrocytes, perhaps in conjunction with BMP-activated Smad transcription factors. It is indeed noteworthy that the pattern of expression of *Cbfa1/Runx2* is modified in *Dlx5/6^{-/-}* mutant mouse embryos (Robledo *et al.*, 2002).

Several observations suggest that it is also possible that *Dlx5* may interact or cooperate with β -catenin-mediated Wnt signaling in positively regulating chondrocyte maturation.

tion. The effects of retroviral misexpression of Wnt-4 or activated β -catenin during skeletal development in the chick limb (Hartmann and Tabin, 2000) are quite similar to those we observe as a consequence of misexpression of *Dlx5*. Misexpression of each not only promotes chondrocyte maturation resulting in the formation of shortened skeletal elements containing excess numbers of hypertrophying chondrocytes, but misexpression of each also stimulates periosteal bone formation. These similar phenotypic effects suggest there may be a direct regulatory relationship between β -catenin-mediated Wnt signaling and *Dlx5* in the positive control of chondrocyte maturation. Thus, it is noteworthy that the expression of *Dlx* genes is regulated by β -catenin-mediated Wnt signaling in some developing systems (Neumann and Cohen, 1997; Beanan *et al.*, 2000). In particular, during development of the wing imaginal disc in *Drosophila*, *Distal-less*, the *Drosophila* homolog of vertebrate *Dlx* genes, is a target gene of Wingless, the *Drosophila* homolog of Wnt-1, which signals through the canonical β -catenin pathway ((Neumann and Cohen, 1997). Taken together, these results suggest the possibility that *Dlx5* might participate in mediating at least some of the effects of the β -catenin Wnt signaling pathway during chondrocyte maturation, and/or that the Wnt signaling pathway and *Dlx5* may work together in regulating the process.

Dlx5 May Also Be Involved in Regulating Osteoblast Differentiation during Formation of the Periosteal Bony Collar

In addition to promoting chondrocyte maturation in the cartilage models, *Dlx5* misexpression enhances periosteal bone formation. Conversely, periosteal bone formation is severely reduced and retarded in *Dlx5*^{6-/-} mutant mouse embryos (Robledo *et al.*, 2002). The increase in periosteal bone we observe as a result of *Dlx5* misexpression could result from *Dlx5* directly promoting enhanced osteoblast differentiation from osteogenic precursor cells in the periosteum, or it may be a secondary consequence of the enhanced chondrocyte maturation that is occurring resulting in enhanced osteogenic signaling from the maturing chondrocytes to the periosteum. Although the latter possibility cannot be excluded, several observations suggest that *Dlx5* may play a direct role in regulation of osteoblast differentiation during formation of the periosteal bony collar.

During normal development, *Dlx5* is highly expressed by the perichondrium adjacent to the diaphysis of the models that will give rise to the osteoblasts that deposit the periosteal bony collar. As endochondral ossification proceeds, *Dlx5* is indeed very highly expressed in the inner layers of the periosteum, where osteoblasts are differentiating and depositing periosteal bone. *Dlx5* is also expressed by differentiating osteoblasts during intramembranous ossification (Simeone *et al.*, 1994; Zhao *et al.*, 1994). Furthermore, we have found that retroviral overexpression of *Dlx5* in primary cultures of chick embryo calvarial cells dramati-

cally accelerates osteoblast differentiation and prematurely induces expression of several osteoblast markers (Tadic *et al.*, 2002). *Dlx5* also stimulates osteoblastic differentiation of calvarial periosteal cells that do not normally undergo osteoblastic differentiation *in vitro* (Tadic *et al.*, 2002). Overexpression of *Dlx5* also upregulates production of *osteocalcin* and mineralization in MC3T3 osteoblast cells (Miyama *et al.*, 1999). *Dlx5* has been directly implicated in the transcriptional activation of several osteoblast genes, including *Col1a1* (Tadic *et al.*, 2001), *bone sialoprotein* (Benson *et al.*, 2000), and *osteocalcin* (Newberry *et al.*, 1998). Taken together, these observations suggest that *Dlx5* may be directly involved in regulating osteoblast differentiation during the formation of the periosteal bony collar, as well as in regulating chondrocyte maturation.

It is possible that the increase in periosteal bone in response to *Dlx5* misexpression could result in part from decreased turnover or remodeling of periosteal bone. However, an increase in periosteal bone formation is detectable in *Dlx5*-infected skeletal rudiments as early as day 13, which is before significant remodeling and turnover of the periosteal bone at its inner (endosteal) surface normally occurs (Pechak *et al.*, 1986b).

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